MALACOLOGIA, 2014, 57(2): 365-376

CALCIUM DETECTION AND OTHER CELLULAR STUDIES
IN THE ESOPHAGUS AND CROP OF THE MARINE SLUG
AGLAJA TRICOLORATA (EUOPISTHOBRANCHIA, CEPHALASPIDEA)

Alexandre Lobo-da-Cunha12", Joana Pereira-Sousa2, Elsa Oliveira’, Angela Alves’, Fernanda Guimaraes? & Goncalo Calado4

ABSTRACT

To obtain more data about the digestive system of cephalaspideans, the esophagus and crop of Aglaja tricolorata were investigated using light and electron microscopy. In this ce-phalaspidean, the low epithelial cells of the anterior esophagus have a border of microvilli covered by a cuticle formed by acid polysaccharides. The esophageal pouches at the hind region of the anterior esophagus contain subepithelial secretory cells filled with electron-dense vesicles rich in neutral polysaccharides and proteins. A ciliated epithelium covers the base of the esophageal pouches. In the crop, the tall columnar epithelium is covered by a cuticle and forms many deep invaginations. The cuticle on the surface of the epithelium contains acid polysaccharides, but the material filling the lumen of the epithelial invaginations consists of neutral polysaccharides and proteins. Epithelial cells of the crop have a border of short microvilli and many mitochondria; lysosome-like bodies and some lipid droplets are present in the apical region. The elongated nucleus, numerous deep cell membrane invaginations and several hemidesmosomes are located at the basal region. Secretory cells are absent in the crop. Numerous large vacuolar cells are present in the connective tissue of the crop. With the pyroantimonate method, calcium was detected in the peripheral vesicles and central vacuole of these cells. WDS X-ray Intensity maps obtained by EPMA (Electron microprobe) confirmed the presence of calcium. This result supports the relationship between the vacuolar cells of aglajids and the calcium cells of pulmonate gastropods. In the posterior esophagus, epithelial cells are similar to the epithelial cells of the crop and secretory cells are also absent.
Keywords: Aglajidae, digestive tract, Gastropoda, histochemistry, microscopy, calcium
cells, microanalysis.

INTRODUCTION

Currently, opisthobranch research is largely
devoted to molecular phylogenetic studies
(Klussmann-Kolb et al., 2008; Malaquias et al.,
2009; Dinapoli & Klussmann-Kolb, 2010; Jörger et al., 2010; Wagele et al., 2014). As a conse-quence, the taxonomy has been profoundly revised and the clade Euopisthobranchia was recently created to include the Tylodinoidea (or Umbraculoidea), Cephalaspidea, Runcinacea, Anaspidea (or Aplysiomorpha) and Pteropoda (Jörger et al., 2010; Wägele et al., 2014). On the other hand, the digestive system of these gastropods has received much less attention in recent years and is far from being fully in-

vestigated. The clade Cephalaspidea has a worldwide distribution and it is the largest within the Euopisthobranchia, comprising more than 800 species of marine slugs and bubble snails (Wagele, 2004). However, for most species just gross anatomy data are available, but in order to understand the evolution of the diges-tive system in cephalaspideans more detailed information must be obtained from species be-longing to different families/clades. The family Aglajidae consists of carnivorous marine slugs that feed on other opisthobranchs, polychaetes, nemerteans and flat worms, although the spe-cific diet of Aglaja tricolorata is still unknown (Gosliner, 1980; Rudman & Willan, 1998). The digestive system of aglajids includes a bulbous

‘Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, 4050-313 Porto, Portugal 2Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), 4050-123 Porto, Portugal 3LNEG, 4466-956 $. Mamede de Infesta, Portugal
4Lusophone University of Humanities and Technologies, 1749-024 Lisbon, Portugal
*Corresponding author: alcunha@icbas.up.pt

366 LOBO-DA-CUNHA ET AL.

or tubular muscular buccal bulb that can be
eversible in some species to grasp the prey,
but the radula is present only in Odontoglaja
(Gosliner, 1980; Rudman & Willan, 1998).
The muscular gizzard with three hard plates,
which is considered a synapomorphy of the cephalaspideans (Mikkelsen, 1996), is absent in aglajids (Rudman, 1974; Gosliner, 1980).A t least in most aglajids, the prey is swallowed whole and slowly digested within the crop by a fluid containing enzymes secreted by the digestive gland (Rudman, 1972). Subsequently, a nutrient rich fluid proceeds to the stomach embedded in the digestive gland and linked to it by ducts. Endocytosis of nutritive particles and lysosomal digestion take place in the digestive cells of the digestive gland (Rudman, 1972), but also seems to occur in the esophagus and crop of cephalaspideans (Lobo-da-Cunha et al., 2010a, 2010b, 2011), as in other gastropods (Walker, 1972; Bourne et al., 1991; Lobo-da-Cunha & Batista-Pinto, 2005).
Recently, the first histochemical and ultra-structural studies of digestive organs of aglajids were published, revealing formally unknown aspects (Lobo-da-Cunha et al., 2009, 2011). However, additional species must be investi-gated in order to obtain a more comprehensive perspective of the digestive system in this fam-ily. The vacuolar cells found in the connective tissue of the crop in Philinopsis depicta are of special interest. The morphology of these cells suggests a relationship with the calcium cells of pulmonates (Lobo-da-Cunha et al., 2011), and to explore this hypothesis calcium detection methods were applied in the study of the crop of Aglaja tricolorata.

MATERIAL & METHODS

Morphology

Several specimens of Aglaja tricolorata Re-nier, 1807, about 4 cm in length were captured in the Arade Estuary on the south coast of Portugal. For light microscopy, the esophagus and crop were fixed in Bouin’s solution for 24 h, dehydrated with ethanol and embed-ded in paraffin. Tissue sections were stained with hematoxylin and eosin, or with Masson’s trichrome. Small pieces of these organs (no more than 1 mms) were fixed for about 2 h at 4°C in fixative containing 2.5% glutaralde-hyde and 4% formaldehyde (obtained from hydrolysis of paraformaldehyde) diluted with

0.4 M cacodylate buffer pH 7.4 (final buffer concentration 0.28 M). After washing in buffer, tissue fragments were post-fixed with 2% OsO, buffered with cacodylate, dehydrated in ethanol and embedded in Epon. Semithin sections for light microscopy were stained with methylene blue and azure Il. Ultrathin sections for trans-mission electron microscopy were stained with uranyl acetate and lead citrate, before being observed in a JEOL 100СХИ transmission electron microscope operated at 60 kV.

Histochemistry

The tetrazonium coupling reaction for protein detection, PAS reaction for polysaccharides, and alcian blue staining for acid polysaccharide detection were applied to deparaffinised sec-tions of esophagus and crop fixed with Bouin’s solution (Ganter & Jolles, 1970). The same techniques were used in semithin sections (2 um) of Epon embedded pieces, but for alcian blue staining the embedding medium was removed by treatment of the sections with a saturated solution of NaOH in ethanol for 10 min (Lane & Europa, 1965).

Tetrazonium Coupling Reaction — Sections were treated for 10 min with a freshly pre-pared 0.2% solution of fast blue salt B in veronal-acetate buffer pH 9.2, washed and treated for 15 min with a saturated solution of B-naphthol in veronal-acetate buffer pH 9.2.

PAS - Sections were treated with 1% periodic acid for 10 min, washed and stained with Schiffs reagent for 15 min. In control sections the oxidation with periodic acid was omitted.

Alcian Blue — Sections were stained with a 0.5% solution of alcian blue diluted in 3% acetic acid at pH 2.5 for detection of car-boxylated polysaccharides and in an HCl solution with pH 1.0 for detection of sulphated polysaccharides.

Calcium Detection

Crop fragments no bigger than 1 mms: were fixed with 2.5% glutaraldehyde and 4% formal-dehyde (obtained from hydrolysis of paraformal-dehyde) diluted in 0.2 M phosphate buffer pH 7.4 containing 1% sucrose, for 12 h at 4°C (adapted from Probst, 1986). For fixation of control frag-ments, 10 mM of EGTA were added to this fixative. After washing in phosphate buffer for at

THE ESOPHAGUS AND CROP OF AGLAJA TRICOLORATA 367

least 2 h, tissue fragments were post-fixed for 2 h at 4°C with an unbuffered solution containing 2% OsO, and 2% potassium pyroantimonate, dehydrated in ethanol and embedded in Epon (Wick & Hepler, 1932). Ultrathin sections were stained for 10 min with uranyl acetate. To confirm the presence of calcium in the electron-dense deposits, ultrathin sections were treated for 30 min at 60°C with a 10 mM solution of EGTA. To prepare this solution the pH was raised to 9-10 with NaOH, and after dissolution of EGTA the pH was lowered to 7.2 with HCI (Probst, 1986). For controls, ultrathin sections were incubated under the same conditions with water at pH 7.2. Ad-ditionally, 4 um thick semithin sections mounted on squares of aluminum foil were carbon coated to provide a conductive layer on the surface and prevent charging. A JEOL JXA 8500-F micro-probe was used to detect the presence of Ca and its spatial distribution. X-Ray Intensity maps were obtained by WDS spectrometers equipped with a PETH crystal for Ca (Ka) line and PETJ crystal for Sb (La) line.

RESULTS

Anatomy, Histology and Histochemistry

The anterior part of the digestive tract of A. tricolorata includes a muscular buccal bulb, a short anterior esophagus connected to the crop, followed by the posterior esophagus (Fig. 1). In the observed animals, the crop was empty or contained unrecognizable food remains.

At the connection between the anterior esophagus and the crop, depressions on the di-gestive tract wall form the esophageal pouches (Figs. 2, 3). Eosinophilic subepithelial secre-tory cells are present around the esophageal pouches, on their lateral walls and base, but were not observed in the remaining part of the anterior esophagus (Figs. 2, 3). These cells are filled with secretory vesicles that are not stained by alcian blue (Fig. 4), but strongly stained by PAS and tetrazonium coupling reaction (Figs. 5-7). The apical region of these cells crosses the epithelium and their nuclei are located in the basal region that is inserted in the connec-tive tissue (Fig. 7). The low epithelium of the anterior esophagus and lateral walls of the pouches are covered by a cuticle stained by al-cian blue (Fig. 4), but not by PAS or tetrazonium coupling reaction (Figs. 5, 6). The bases ofthe esophageal pouches are covered by a ciliated epithelium without cuticle, but secretory cells ofthe same type are present (Fig. 7).
The tall columnar epithelium ofthe crop forms many deep, branched invaginations, but secre-tory cells are absent (Figs. 8, 9). The crop epi-thelium is covered by a cuticle strongly stained by hematoxylin, and the lumen о the epithelial invaginations is also stained by hematoxylin (Fig. 9). On the surface oft he epithelium the cu-ticle is not stained by the tetrazonium coupling reaction, but a positive reaction is detected in
the lumen of the epithelial invaginations (Fig.
10). Conversely, the cuticle on the surface of
the epithelium is stained by alcian blue, but acid
polysaccharides are not detected in the lumen

—У

FIGS. 1-12. Anatomy, histology and histochemistry. FIG.1: Anatomy of the digestive system. bb — buccal bulb, sg — salivary glands, ae — anterior esophagus, cr — crop, pe — posterior esophagus, dg — digestive gland; FIG. 2: Esophageal pouch (arrow) at the transition zone between the anterior esophagus (ae) and the crop (cr). Eosinophilic secretory cells (arrowheads) are present in this zone. Section stained with haematoxylin and eosin; FIG. 3: Semithin section of an esophageal pouch. Secretory cells are found at the base (arrow) and in the lateral walls of the pouch (arrowheads); FIG. 4: The cuticle covering the epithelium of the esophageal pouches is stained by Alcian blue (arrowheads), but the secretory cells (sc) are not stained; FIG. 5: The secretory cells of the esophageal pouches (sc) are strongly PAS positive, but the cuticle (arrowhead) is unstained; FIG. 6: The cuticle of the esophageal pouches (arrowhead) is not stained by the tretrazonium coupling reaction, but the secretory cells (sc) are strongly stained; FIG. 7: Secretory cell (sc) at the base of an esophageal pouch stained by the tetrazonium coupling reaction. The nucleus (nu) is located in the basal region of the cell that is embedded in the connective tissue, and the apical region crosses the ciliated epithelium (asterisk); FIG. 8: Section of the crop stained with Masson’s trichrome, showing the epithelium (arrows) and several vacuolar cells in the connective tissue (arrowheads); FIG. 9: Section of the crop stained with hematoxylin and eosin. The epithelium forms deep invaginations (arrows) and is covered by a cuticle that stains with hematoxylin (arrowheads). Several vacuolar cells (asterisks) are visible in the connective tissue; FIG. 10: The cuticle of the crop (arrowhead) is not stained by the tetrazonium coupling reaction, but the lumen of the epithelial invaginations shows positive reaction (arrows); FIG. 11: The cuticle of the crop (arrowheads) is stained by alcian blue, but the lumena of the epithelial invaginations are not (arrows); FIG. 12: In the crop, the cuticle (arrowheads) and the lumena of the epithelial invaginations (arrows) are both strongly PAS positive.

THE ESOPHAGUS AND CROP OF AGLAJA TRICOLORATA 369

FIGS. 13-16. Ultrastructure of the anterior esophagus cells. FIG. 13: Epithelial cells from the lateral walls of an esophageal pouch showing the cuticle (asterisk), microvilli (arrowheads), interdigitations between cells (arrows), lysosome-like bodies (ly) and the nucleus (nu); FIG. 14: Epithelial cells densely covered by cilia (ci) atthe base of an esophageal pouch. Ciliar roots (arrowhead) and nuclei (nu) are also visible; FIG. 15: The depression at the apex of the secretory cells is filled with microvilli (asterisk), and electron-dense secretory vesicles (sv) are abundant in the cytoplasm; FIG. 16: The basal region of the secretory cells contains the nucleus (nu), rough endoplasmic reticulum cisternae (arrowheads), Golgi stacks (arrow) and numerous secretory vesicles (sv) some of them still immature (asterisks).

370 LOBO-DA-CUNHA ET AL.

FIGS. 17-20. Ultrastructure of the crop and posterior esophagus cells. FIG. 17: Crop epithelial cells possess a border of microvilli (arrowheads) covered by a thick cuticle (asterisk). Many mitochondria (mi) and several lysosomal-like bodies (ly) are visible in the supranuclear region; FIG. 18: Nuclei (nu) at the basal region of crop epithelial cells; FIG: 19. Cell membrane invaginations (arrowheads) are abundant in the basal region of crop epithelial cells. À hemidesmosome (arrow) attached to a bundle of intermediate filaments is also visible. bl — basal lamina; FIG. 20: Apical region of the epithelial cells from the posterior esophagus showing the cuticle (asterisk), microvilli (mv), mitochondria (mi) and lysosome-like bodies (ly).

THE ESOPHAGUS AND CROP OF AGLAJA TRICOLORATA 371

of the epithelial invaginations (Fig. 11). PAS positive material was detected both on the sur-face of the epithelium and in the invaginations (Fig. 12). In the connective tissue of the crop many very large vacuolar cells are present, mostly near the epithelium, being surrounded by muscle cells (Figs. 8, 9).

Ultrastructure and Microanalysis

In the anterior esophagus and lateral walls
of the esophageal pouches the epithelial cells
have a border of short microvilli covered by
a thin cuticle. These cells contain several
lysosome-like structures and many interdigita-

FIGS. 21-25. Ultrastructure and calcium detection in vacuolar cells. FIG. 21: Vacuolar cell with a thin peripheral cytoplasmic layer (arrowheads) and several structures with ring-shaped cross-section (arrows) inside the vacuole (va); FIG. 22: Structures with ring-shaped cross-section are also present within vesicles (ve) in the peripheral cytoplasm of the vacuolar cells; FIG. 23: Tubular cell membrane invagination (ar-row) in a vacuolar cell. A thick basal lamina (bl) surrounds these cells; FIG. 24: Calcium detection with pyroantimonate in a vacuolar cell. Electron-dense deposits (arrows) are noticeable in the vacuole (va) and in vesicles (ve). Other cell types contain much lower amounts of electron-dense antimonate deposits (arrowhead); FIG. 25: Electron-dense antimonate deposits were removed from ultrathin sections after treatment with 10 mM EGTA for 30 min at 60°C, leaving clear spots in the epoxy resin (arrows).

312 LOBO-DA-CUNHA ET AL.

tions connect neighboring cells (Fig. 13). At
the base of the esophageal pouches, epithelial
cells possess a border of cilia and the cuticle
is absent (Fig. 14). The secretory cells of the
esophageal pouches contain a large number of electron-dense vesicles, attaining a diameter of 1.5 um. The apical region of these cells forms a depression that is full of microvilli (Fig. 15). The basal region, which is inserted into the con-nective tissue, contains endoplasmic reticulum cisternae around the nucleus and a few Golgi stacks are also present nearby (Fig. 16). Epithelial cells of the crop have a border of short microvilli covered by a thick cuticle. Ad-ditionally, in the apical region these cells contain many mitochondria, lysosome-like bodies and some lipid droplets (Fig. 17). The elongated nucleus is basally located and numerous deep cell membrane invaginations are formed in the basal region (Figs. 18, 19). Several hemidesmo-somes attached to bundles of intermediate fila-ments were observed on the basal membrane

(Fig. 19). In the posterior esophagus, epithelial cells are similar to the epithelial cells of the crop (Fig. 20), and secretory cells are not present. The vacuolar cells of the crop connective tissue are characterized by a very thin layer of cytoplasm surrounding a huge vacuole that occupies most of the cell volume. Several structures showing a ring-shape when viewed in cross-section were found inside the vacu-ole and also within vesicles located in the peripheral cytoplasm (Figs. 21, 22). Tubular cell membrane invaginations are another trait of these cells (Fig. 23). Calcium detection with potassium pyroantimonate revealed that these cells contain significant amounts of calcium. A large quantity of electron-dense antimonate deposits were observed in the central vacuole and in vesicles (Fig. 24), although some vacu-olar cells contained more deposits than others. Small electron-dense deposits were also visible in other cell types, including muscle cells. Addi-tion of EGTA during fixation of control fragments

EesRHrRNG=SEaHSBSOa) T ee O l
=

o.
в.
6.
8
о.
0.
0.
6.
0.

0
0
0
о
о
0
1
1
3
7
5
7
5
6
3

4
.0
0

=

ыы
DUcNabeOwteol

FIGS. 26-28. Microanalysis in vacuolar cells. FIG. 26: Backscattered electron image of a semithin sec-tion showing extensive antimonate deposits (arrows) in vacuolar cells (va). mc — muscle cells; FIG. 27: Intensity X-ray distribution map of calcium in the same semithin section. Calcium is more abundant in the areas corresponding to the antimonate deposits in vacuolar cells (arrows); FIG. 28: Intensity X-ray distribution map of antimony shows that the areas with higher amounts of antimony (arrows) correspond to the areas with higher amounts of calcium.

THE ESOPHAGUS AND CROP OF AGLAYA TRICOLORATA AS

TABLE 1. Summary of main features of the esophagus and crop of A. fricolorata and P. depicta.

Crop histology

Anterior
esophagus

Species

A. tricolorata Short, with esopha-Invaginations
geal pouches
P. depicta Absent Ridges

was not sufficient to prevent the formation of electron-dense deposits. However, the anti-monate deposits were completely removed from ultrathin sections treated with 10 mM EGTA for 30 min at 60°C (Fig. 25), confirming the presence of calcium. The electron-dense deposits were not affected in water-incubated ultrathin sections. Additionally, 4 um thick semi-thin sections were analyzed with an electron microprobe equipped with WDS detectors. Vacuolar cells containing electron-dense de-posits were clearly visible in backscattered electron images obtained with the microprobe (Fig. 26). Although low amounts of calcium were detected (Fig. 27), the distribution of this element in the sections correlates well with the distribution of antimony (Fig. 28), corroborat-ing the presence of calcium in the antimonate electron-dense deposits.

DISCUSSION

Among aglajids, histochemical and ultra-structural data on the esophagus and crop are available only for A. tricolorata (present article) and Philinopsis depicta (Lobo-da-Cunha et al., 2011). Acomparison between these species re-veals substantial differences, but also similari-ties in the digestive tract (Table 1). Contrary to what happens in А. tricolorata, in Р depicta an anterior esophagus is not noticeable between the buccal bulb and the crop (Lobo-da-Cunha et al., 2011). In P depicta, the crop wall forms many ridges and two large folds (Lobo-da-Cunha et al., 2011). However, in A. tricolorata instead of ridges the crop presents numerous deep and branched invaginations, which are a different way to obtain an enlargement of the epithelial surface and enhance absorption
of substances from the lumen. Another differ-
ence between these species is the presence
of mucus-secreting cells in the crop epithelium

Epithelium Secretory Vacuolar cells cells

Absent

Present

Histology of the posterior
esophagus

Present Identical to crop, but with ridges instead of invaginations
Present Identical to crop

of P. depicta and its absence in A. tricolorata. Moreover, the secretory cells found in the esophageal pouches and surrounding areas of the anterior esophagus of A. tricolorata secrete proteins associated with neutral polysaccha-rides, whereas the crop secretory cells of Р depicta secrete acid polysaccharides (Lobo-da-Cunha et al., 2011). Among all of these dif-ferences, the presence of many vacuolar cells in the connective tissue of the crop wall in both species seems to have a special significance. The presence of these peculiar cells in such large numbers in the two genera of aglajids that have been studied by transmission electron mi-croscopy suggests that this could be a special trait of the crop of aglajids. So far, similar cells have not been reported in the digestive tract of other euopisthobranchs (Lobo-da-Cunha & Batista-Pinto, 2005; Lobo-da-Cunha et al., 2010a, 2010b).
The vacuolar cells of the crop connective tissue have the same main features in A. tricolorata and P. depicta, which are the large vacuole occupying most of the cell volume and the tubular cell membrane invaginations. However, in A. tricolorata the central vacuole and peripheral vesicles contain structures with a ring-shaped cross-section that were not observed in the vacuolar cells of P. depicta (Lobo-da-Cunha et al., 2011). Previously, it was suggested that these vacuolar cells could be related to the calcium cells of pulmonate gastro-pods (Lobo-da-Cunha et al., 2011), which could be a specific and modified type of rhogocyte according to Haszprunar (1996). In pulmonate gastropods, calcium cells reach a large diam-eter and contain large vacuoles loaded with concretions mainly consisting of calcium car-bonate deposited on an organic matrix (Sminia et al., 1977; Luchtel et al., 1997). These cells can contain a single vacuole surrounded by a very thin layer of cytoplasm, with a peripheral nucleus (Richardot & Wautier, 1972; Sminia

374 LOBO-DA-CUNHA ET AL.

et al., 1977), resembling the vacuolar cells of
aglajids. The typical calcium concretions of
mollusks present a multilayered concentric
pattern, but in calcium cells of Lymnaea stag-
nalis some oft hese formations show a simpler
structure consisting on a single electron-dense layer (Sminia et al., 1977), closely resembling the structures with a ring-shaped cross-section found in A. tricolorata vacuolar cells. The py-roantimonate method revealed the presence of calcium in vacuolar cells of A. tricolorata, sup-porting the relationship with the calcium cells of pulmonates. However, the electron-dense antimonate deposits are scattered all over the central vacuole and vesicles, but not associ-ated with the ring-shaped formations. Control with the calcium chelator EGTA added during fixation might not be effective in preventing the formation of electron-dense deposits contain-ing calcium (Probst, 1986). For this reason, incubation of ultrathin sections with EGTA was used to confirm the presence of calcium. This last procedure specifically removes calcium causing the dissolution of the electron-dense deposits (Wick & Hepler, 1982; Probst, 1986), as reported in the vacuolar cells of A. tricolo-rata. An additional confirmation ofthe presence of calcium in the antimonate deposits was ob-tained with WDS X-Ray mapping of the cells. Experiments with the freshwater pulmonate L. stagnalis under acidosis conditions suggest that calcium cells can be a source of ions for pH buffering (Sminia et al., 1977), and that is a possible function for the connective tissue vacuolar cells in the crop of aglajids.
In A. tricolorata, a cuticular layer covers the epithelium of the esophagus and crop. How-ever, the histochemical analysis shows that these cuticles have differences in composition. In the anterior esophagus the cuticle contains acid polysaccharides that are not PAS reac-tive, whereas the cuticle covering the crop epithelium contains acid polysaccharides and is stained by PAS reaction. More significant are the histochemical differences between the cuticle on the surface of crop epithelium and the material filling the lumen of crop epithelial invaginations. Both contain PAS reactive poly-saccharides, but acid polysaccharides are only detected in the cuticle covering the surface of the epithelium in direct contact with the lumen. On the other hand, proteins that are reactive to the tetrazonium coupling reaction are found in the lumen of the invaginations but not on the surface of the crop epithelium. Acid polysac-charides were also detected in the extracel-

lular material covering the crop epithelium of P. depicta, but in this species the epithelium does not form invaginations (Lobo-da-Cunha et al., 2011). However, it seems that the extracel-lular materials covering these epithelia can be crossed by substances that are collected by epithelial cells. In gastropods, esophageal and crop epithelial cells typically possess several lysosomes in the cytoplasm above the nucleus (Bush, 1989; Leal-Zanchet, 2002; Lobo-da-Cunha & Batista-Pinto, 2005; Lobo-da-Cunha et al., 2010a), which seem to be involved in the intracellular digestion of substances collected by endocytosis (Boer & Kits, 1990; Bourne et al., 1991), and the abundance of lysosome-like bodies in the crop epithelium of A. tricolorata suggests a similar activity. Additionally, the numerous deep cell membrane invaginations in the basal region of crop epithelial cells in A. tricolorata are a sign of intense transport activ-ity, also reported in other gastropods (Payne & Crisp, 1989). All these aspects indicate that the crop epithelium performs an important physiological role.
Hemidesmosomes are frequent in the crop epithelium of A. triclorata and P-depicta (Lobo-da-Cunha et al., 2011). These cell junctions reinforce the adhesion between epithelial cells and the underlying connective tissue (Bairati & Gioria, 2008) and might be particularly im-portant in the crop of aglajids that is subjected to substantial distention when filled with food (Lobo-da-Cunha et al., 2011).
In cephalaspideans with gizzard, a dilated crop is not present, and the gizzard divides the esophagus into anterior and posterior regions (Lobo-da-Cunha et al., 2010a, 2010b). Aglajids have lost the gizzard and acquired a dilated crop. Considering that in A. tricolorata and P. depicta the crop and the posterior esophagus present very similar histological and ultrastruc-tural aspects, and given that in A. tricolorata the anterior esophagus is substantially dif-ferent from the crop, according to available data, it seems that the crop of aglajids was originated as a dilated region of the posterior esophagus.
The anatomical and functional characteristics of the buccal bulb have been considered the most significant features of the digestive tract for distinction among aglajid genera (Rudman, 1974; Gosliner, 1980). However, detailed in-formation on the digestive system of aglajids and other cephalaspideans is still scarce. In most taxonomic articles, the digestive system is very briefly described, but the new stud-

THE ESOPHAGUS AND CROP OF AGLAJA TRICOLORATA 375

les on the esophagus and crop of P depicta (Lobo-da-Cunha et al., 2011) and A. tricolorata have revealed substantially different aspects that should be investigated in other species to evaluate potential taxonomic relevance.

ACKNOWLEDGEMENTS

The authors thank Mr Joäo Carvalheiro and Miss Joana Carvalheiro for the reproduction of photomicrographs. This work was partially supported by ICBAS-UP and by the European Regional Development Fund (ERDF) through the COMPETE - Operational Competitiveness Programme and national funds through FCT — Foundation for Science and Technology, under the project PEst-C/MAR/LA0015/2011.

LITERATURE CITED

BAIRATI, A. & М. GIORIA, 2008, An ultrastructural study of cell junctions and the cytoskeleton in epithelial cells of the molluscan integument. Journal of Morphology, 269: 319-331.
BOER, H. H. & K. S. KITS, 1990, Histochemical and ultrastructural study of the alimentary tract of the freshwater snail Lymnaea stagnalis. Journal of Morphology, 205: 97-111.
BOURNE, N. B., G. W. JONES & I. D. BOWEN, 1991, Endocytosis in the crop of the slug, Deroceras reticulatum (Muller) and the effects of the ingested molluscicides, metaldehyde and methiocarb. Journal of Molluscan Studies, 57: 71-80.
BUSH, M. S., 1989, The ultrastructure and function of the esophagus of Patella vulgata Linnaeus. Journal of Molluscan Studies, 55: 111-124.
DINAPOLI, A. & A. KLUSSMANN-KOLB, 2010, The long way to diversity — Phylogeny and evolution of the Heterobranchia (Mollusca: Gastropoda). Molecular Phylogenetics and Evolution, 55: 60-76. _
GANTER, P. & G. JOLLES, 1970, Histochimie normal et pathologique. Gauthier-Villars, Paris, 2 vols.
GOSLINER, T. M., 1980, Systematics and phylogeny of the Aglajidae (Opisthobranchia: Mollusca). Zoological Journal of the Linnean Society, 68: 325-360.
HASZPRUNAR, G., 1996, The molluscan rhogo-cyte (pore-cell, blasenzelle, cellule nucale), and its significance for ideas on nephridial evolution. Journal of Molluscan Studies, 62: 185-211. JORGER, К. M., |. STOGER, Y. KANO, Н. FUKU-DA, T. KNEBELSBERGER & M. SCHRODL, 2010, On the origin of Acochlidia and other enigmatic euthyneuran gastropods, with impli-cations for the systematics of Heterobranchia. BMC Evolutionary Biology, 10(323): 1-20.

KLUSSMANN-KOLAB., A.D INAPOLI, K. KUHN, B. STREIT & C. ALBRECHT, 2008, From sea to land and beyond — New insights into the evolu-tion of euthyneuran Gastropods (Mollusca). BMC Evolutionary Biology, 8(57): 1-16.
LANE, B. P. & D. L. EUROPA, 1965, Differential staining of ultrathin sections of Epon-embedded tissues for light microscopy. Journal of His-tochemistry and Cytochemistry, 13: 579-582. LEAL-ZANCHET, A. M., 2002, Ultrastructure of the supporting cells and secretory cells of the alimentary canal of the slugs, Lehmannia marginata and Boettgerilla pallens (Pulmonata: Stylommatophora: Limacoidea). Malacologia, 44: 223-239.
LOBO-DA-CUNHA, A. & C. BATISTA-PINTO, 2005, Light and electron microscopy studies of the esophagus and crop epithelium in Aplysia depilans (Mollusca, Opisthobranchia). Tissue & Cell, 37: 447—456.
LOBO-DA-CUNHA, A., |. FERREIRA, К. COELHO & G. CALADO, 2009, Light and electron mi-croscopy study of the salivary glands of the Carnivorous opisthobranch Philinopsis depicta (Mollusca, Gastropoda). Tissue & Cell, 41: 367-375.
LOBO-DA-CUNHA, A., Е. OLIVEIRA, A. ALVES, R. COELHO & G. CALADO, 2010a, Light and electron microscopic study of the anterior esophagus of Bulla striata (Mollusca, Opistho-branchia). Acta Zoologica, 91: 125-138. LOBO-DA-CUNHA, A., Е. OLIVEIRA, 1. FER-REIRA, R. COELHO & G. CALADO, 2010b, Histochemical and ultrastructural characterisa-tion of the posterior esophagus of Bulla striata (Mollusca, Opisthobranchia). Microscopy and Microanalysis, 16: 688-698.
LOBO-DA-CUNHA, A., T. SANTOS, Е. OLIVEIRA, A. ALVES, R. COELHO & G. CALADO, 2011, Microscopical study ofthe crop and oesophagus of the carnivorous opisthobranch Philinopsis depicta (Cephalaspidea, Aglajidae). Journal of Molluscan Studies, 77: 322-331.
LUCHTEL, D. L., А. W. MARTIN, I. DEYRUP-OLSEN & H. H. BOER, 1997, Gastropoda: Pulmonata. Pp. 459-718, in: F. W. HARRISON & А. J. Конм, eds., Microscopic anatomy of inver-tebrates, Vols. 6A-B [Mollusca II]. Wiley-Liss, New York, 828 pp.
MALAQUIAS, M. A. E., J. M. DODDS, P. BOUCHET & D. G. REID, 2009, A molecular phylogeny of the Cephalaspidea sensu lato (Gastropoda: Euthyneura): Architectibranchia redefined and Runcinacea reinstated. Zoologica Scripta, 38: 23-41.
MIKKELSEN, P. M., 1996, The evolutionary rela-tionships of Cephalaspidea s. |. (Gastropoda: Opisthobranchia): a phylogenetic analysis. Malacologia, 37: 375—442.
PAYNE, C. M. & M. CRISP, 1989, Ultrastructure and histochemistry of the posterior esophagus of Nassarius reticulatus (Linnaeus). Journal of Molluscan Studies, 55: 313-321.
PROBST, W., 1986, Ultrastructural localization of calcium in the CNS of vertebrates. Histochemis-try, 85: 231-239.

376 LOBO-DA-CUNHA ET AL.

RICHARDOT, M. & J. WAUTIER, 1972, Les cellules
à calcium du conjonctif de Ferrissia wautieri (Moll.
Ancylidae). Description, minéralogie et variation
saisonnieres. Zeitschrift für Zellforschung, 134:
227-243.
RUDMAN, W. B., 1972, Structure and functioning ofthe gut in the Bullomorpha (Opisthobranchia). Part 4. Aglajidae. Journal of Natural History, 6: 547-560.
RUDMAN, W. B., 1974, A comparison of Cheli-donura, Navanax and Aglaja with other genera ofthe Aglajidae (Opisthobranchia: Gastropoda). Zoological Journal of the Linnean Society, 54: 185-212.
RUDMAN, W. В. & R. С. WILLAN, 1998, Opist-hobranchia. Pp. 915-1035, in: P. L. BEESLEY, G. J. В. Ross & А. WeLLs, eds., Fauna of Austra-lia. The southern Synthesis, Vol. 5. Mollusca. CSIRO Publishing, Melbourne, xv + 1234 pp. SMINIA, Т., N. D. DE WITH, J. L. BOS, М. Е. VAN NIEUWMEGEN, M. P. WITTER & J. WONDERGEM, 1977, Structure and function of the calcium cells of the freshwater pulmonate

snail Lymnaea stagnalis. Netherlands Journal of Zoology, 27: 195-208.
WAGELE, H., 2004, Potential key characters in Opisthobranchia (Gastropoda, Mollusca) en-hancing adaptive radiation. Organism Diversity and Evolution, 4: 175-188.
WAGELE, H., A. KLUSSMANN-KOLB, E. VER-BEEK & M. SCHRODLE, 2014, Flashback and foreshadowing - a review of the taxon Opistho-branchia. Organisms Diversity and Evolution, 14: 133-149.
WALKER, G., 1972, The digestive system of the slug, Agriolimax reticulatus (Müller): experi-ments on phagocytosis and nutrient absorption. Proceedings of the Malacological Society of Lond4o0: n33,—43 .
WICK, S. M. & P. K. HEPLER, 1982, Selective localization of intracellular Ca2+ with potassium antimonate. Journal of Histochemistry and Cy-tochemistry, 30: 1190-1204.

Revised ms. accepted October 7, 2013